

Mutagen Formation during Commercial Processing of Foods

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Levels of bacterial mutagenicity 3–17 times above spontaneous are generated during commercial thermal processing (canning) of foods, particularly foods high in protein. The potential for other processing operations, including pasteurization, dehydration, and concentration, to produce substances active in the Ames Salmonella assay was also examined. Two heated fish model systems, canned salmon and fried sole, were established by extracting mutagen precursors from fish tissues with water. The model system studies suggest that the limiting reactants for mutagen formation differ from one food product to another, and that Maillard type browning reactions are involved in mutagen production. Bisulfite treatment was found to inhibit mutagen formation in model systems and whole food products.

Isolation and partial characterization of the mutagens in both fried and canned pink salmon showed that at least three distinct mutagens were present. These mutagens exhibited HPLC retention time patterns on C₁₈, cyano, and amino columns different than the major mutagens present in other cooked and grilled meats and fish.

Introduction

The role of environmental carcinogens in human cancer has been the subject of considerable scientific research in recent years. Foods are such a universal factor in the human survival experience that it is not surprising that the question of potential human carcinogens in foods has been of widespread interest. This symposium has addressed primarily the formation, detection, and identification of mutagens in cooked or broiled foods, especially in meat and fish products. Recent reviews have summarized the work of numerous investigators showing that potent mutagens are formed during the cooking of foods (1–5).

In many cases the structures of the substances responsible for the mutagenic activity have been elucidated and fall mainly into the category of nitrogen-heterocyclic polyaromatic amines. The levels of these compounds found in cooked foods are generally low, in the parts per billion range.

These mutagens are formed not only when foods are heated to the nearly pyrolytic temperatures (> 300°C)

which occur during gas flame grilling but also in the course of more moderate heating processes such as frying, roasting, or baking, where temperatures between 150 and 250°C are common. Foods encounter these same temperatures during many commercial food processing operations; however, little attention has been focused on the influence of these processes on mutagen formation.

This paper summarizes information available on the presence of mutagens in commercially processed foods, examines mutagen formation, its inhibition by specific additives, and the nature of mutagen precursors. It also discusses the partial characterization of mutagens formed during thermal processing.

Occurrence of Mutagens in Processed Foods

Heat is applied to food products during many commercial food processing operations including those listed in Table 1. The temperatures to which foods are exposed can range from less than 100°C to greater than 150°C.

Pasteurization

This process usually involves heating a product (e.g. milk or liquid egg products) to 60 to 80°C for up to a few minutes to destroy pathogenic microorganisms. The short times and relatively low temperatures used

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Table 1. Some commercial food processes which include heat treatments.

Process	Temperature of heating medium, °C	Product temperature, °C
Pasteurization	~100	<100
Concentration	100–125	<100
Open kettle		
Vacuum evaporation		
Drying	100–200	<100
Spray dry		
Drum dry		
Air dry		
Thermal processing	100–135	100–110
Metal cans		
Glass containers		
Retort pouch		

for pasteurization suggest that few mutagens would form. Bjeldanes (6) found no mutagens in pasteurized fresh milk or in milk refluxed up to 240 min. Rogers and Shibamoto (7) were unable to detect mutagens in milk or milk model systems which were heated at 100°C for 2 to 6 hr. Green et al. (8) did report low levels of mutagens (three times the spontaneous rate) in ultra-high-temperature pasteurized milk (135°C for 1 sec, then 20 min at 117°C). However, the histidine content of the milk samples (which were applied directly to the Ames test plates) was not taken into account and may have contributed to the apparent increase in numbers of revertants (9). Thus there is no substantial evidence that pasteurization processes promote mutagen formation.

Batter-Coated and Breaded Foods

Commercially produced batter-coated and breaded convenience foods are heated in vegetable oil at about 200°C prior to packaging and freezing. Browning reactions occur during the heat treatment, with the extent of browning dependent upon time and temperature of heating and composition of the breading. Testing extracts of a variety of batter-coated and breaded products (including fish filets, clams, shrimp, and fish and shrimp sticks) did not reveal the presence of mutagenic substances (10). When these products were subjected to additional heating, as they would be in the home by the consumer, only at twice the manufacturer's recommended times were low levels of mutagenicity found (10). Deep fried batter-coated and breaded products, doughnuts, and potatoes purchased at local restaurants were also found to contain negligible levels of mutagens (6,11).

In these products, the outer portion which receives the most heat input is high in carbohydrate and relatively low in protein. Products of this composition do not tend to form the potent heterocyclic aromatic amine type mutagens as readily as high protein content foods like meats or fish.

Concentration

The evaporative concentration of solutions is one of the most energy intensive unit operations. In the food industry, concentration usually involves evaporation of water, and this can be accomplished by heating in steam-jacketed open kettles or various types of vacuum evaporators. Fruit juices and syrups, dairy products, and meat extracts are frequently concentrated prior to further processing.

Food-grade beef extract is prepared by heating beef tissue in water, removing the meat solids and fat, and concentrating the liquid stock to < 20% of original volume at about 100°C. Potent mutagens have been found to be present in beef extracts (12,13), with IQ (2-amino-3-methylimidazo[4,5-f]quinoline) and MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline) contributing almost 100% of the mutagenic activity in one sample (13). Two commercial products that contained beef extract, bouillon cubes, and a dried gravy mix were also found to contain mutagens (12).

One novel technique for concentration of foods which is currently being investigated at the University of Washington is submerged combustion. This process features the ability to concentrate solutions using temperatures $\leq 75^\circ\text{C}$. An operating submerged combustion unit causes the release of a stream of hot combustion products below the surface of the solution being concentrated. The tremendous number of very small bubbles transfers heat to the solution and carries away water vapor. The product that has been most extensively studied in this submerged combustion system is a fish protein hydrolyzate (FPH) (14). The FPH was produced by digesting minced fish flesh with proteolytic enzymes and, in this study, was concentrated to two levels of soluble solids by submerged combustion prior to spray drying. Extracts of the final products were tested for mutagenicity using the standard plate test described by Ames et al. (15) with and without the

Table 2. Mutagenicity of fish protein hydrolyzates concentrated by submerged combustion at different pHs.

Sample	Mutagenicity activity ratio (MAR) ^a		
	pH	– S9 ^b	+ S9 ^c
Spray dry without concentration	3.5	0.5	2.6
	4.5	0.6	2.5
	6.5	1.1	2.8
Concentrate to 15% solids	3.5	0.5	1.8
	4.5	2.0	59.0
	6.5	0.9	7.2
Concentrate to 25% solids	3.5	toxic	13.0
	4.5	toxic	64.0
	6.5	toxic	6.3

^a *S. typhimurium* TA 1538, basic extracts, the equivalent of 1.6 g dry fish protein hydrolyzate (FPH) per plate. The mutagenic activity ratio (MAR) is calculated by dividing the number of revertants on plates containing extracts by the spontaneous reversion rates.

^b Without metabolic activation.

^c With 80 μL rat liver homogenate preparation per plate.

addition of 80 μ L Aroclor-induced rat liver homogenate preparation (S9) per plate.

The procedure used to prepare the extracts of fish protein hydrolyzate has been described elsewhere (16) and includes homogenization of the product with three volumes of methanol, filtration and removal of the methanol from the filtrate by rotary vacuum evaporation. The methanol-soluble substances were then dissolved in water at pH 2.5, partitioned three times with methylene chloride, and the aqueous phase adjusted to pH 10 with 50% NaOH and again partitioned three times with methylene chloride. The organic extracts were dried over sodium sulfate, the methylene chloride removed by rotary evaporation and the residues dissolved in known volumes of Spectro-grade dimethyl sulfoxide. This procedure, unless specified otherwise, was used throughout the studies reported here to produce acidic and basic extracts for mutagenicity testing.

As shown in Table 2, concentration of FPH by submerged combustion produced high levels of mutagens, particularly when concentration was carried out at pH 4.5. Spray drying without concentration produced little or no mutagenicity. The basic organic extracts contained most of the mutagenicity which was detected only by *S. typhimurium* strain TA 1538 with metabolic activation. No mutagenicity was observed using *S. typhimurium* strain TA 100.

The amino acid patterns of all the FPH were found to be very similar, but when they were included in the diets of rats in order to determine protein efficiency ratios (PER), those concentrated by submerged combustion at pH 4.5 were found to produce significantly lower PERs (14). Whether this was due to toxic factors in the FPH, differences in bioavailability of various nutrients or acceptability of the diet was not determined.

Table 3. Mutagenicity of spray-dried dairy products.

Sample	Extract ^b	MAR ^a	
		- S9 ^c	+ S9 ^d
Instant nonfat dry milk	Acid	Toxic	1.2
	Base	1.6	1.1
Instant 0.5% fat dry milk	Acid	1.5	0.6
	Base	0.7	4.3
Noninstant nonfat dry milk	Acid	Toxic	0.5
	Base	0.8	1.2
Noninstant nonfat dry milk, reconstituted respray-dried	Acid	Toxic	1.1
	Base	0.8	1.2
Whey protein concentrate	Acid	1.2	0.9
	Base	0.7	1.1

^a Mutagenic activity ratio using *S. typhimurium* TA 1538. The MAR is calculated by dividing the number of revertants on plates containing extracts by the spontaneous reversion rates.

^b Methylene chloride extracts equivalent to 5 g dry product per plate.

^c Without metabolic activation.

^d With 80 μ L S9 preparation per plate.

There are several possible explanations for the mutagenicity that was observed in the concentrated samples, including the interaction of food components with reactive combustion products of the flame, even though in theory only CO₂ and H₂O are formed when the fuel is burned. Also since the concentration steps were 60 to 120 min in duration, the types of mutagen formation reactions shown to occur in traditional concentration processes (e.g., production of beef extract) or in model systems may be responsible for mutagenicity in this product. All of the water-soluble components of fish muscle are present in the FPH, and most proteins have been hydrolyzed to amino acids and small peptides, producing high levels of these possible mutagen precursors in the solutions being concentrated.

Dehydration

As mentioned above, the spray-drying process did not seem to produce mutagenic substances. In spray drying, liquid droplets are dispersed as a fine spray into a stream of heated air in a drying chamber. The hot air supplies the heat of vaporization, removes the evaporated moisture, and transports the dried solids to the collection portion of the drier. Dairy products, fruit juices, coffee, and eggs are commonly spray-dried (17). Table 3 lists a variety of commercially spray-dried products and their mutagenicities. Even a spray-dried nonfat milk which was reconstituted and again spray-dried in our pilot plant did not exhibit mutagenicity. These data seem to confirm the observation that spray drying is a process that does not encourage mutagen formation reactions, probably due to the product's short time of exposure to heat.

Many other types of driers are used for dehydration of fruits, vegetables, cereals, pasta products, etc. (18). The driers can be batch (kiln or cabinet) or continuous and use heated air up to ~200°C, or other modes of heat transfer (e.g., infrared) (17,18). Vacuum driers use re-

Table 4. Mutagenicity of some commercially canned meats and seafoods.

Product	MAR ^a	
	- S9 ^b	+ S9 ^c
Pink salmon (brand #1)	0.8	17.6
Beef broth	2.2	13.0
Pink salmon (brand #2)	0.6	11.9
Red salmon	1.3	8.5
Beef stew (retort pouch)	0.9	7.4
Mackerel	1.2	7.2
Roast beef hash	0.4	6.0
Chili with beans	1.1	4.9
Roast beef	2.1	4.6

^a Mutagenic activity ratios for basic extracts from 80 g product using *S. typhimurium* strain TA 1538. Mean spontaneous reversion rate without S9 was 9 and with S9 was 24 revertants. An MAR greater than 2.5 is considered a positive test for mutagenicity.

^b Without metabolic activation.

^c With 80 μ L S9 per plate.

duced pressures and temperatures. During the drying of vegetables and fruits, browning reactions (enzymatic, Maillard type, and carmelization) are likely to occur unless sulfites or other browning inhibitors are applied. In some cases this browning is important for development of desirable sensory attributes, as in raisins, dates, and figs.

At least one-half million tons of dried fruits are produced annually in the U.S. (17). Stich et al. (19) have examined the chromosome-damaging activities of eight widely consumed dried fruits. It was found that aqueous extracts, tested directly or after lyophilization, produced significant increases in chromosome aberrations (exchanges or breaks) in Chinese hamster ovary cells. Whether the substances responsible for these changes present a hazard to humans is unclear.

Commercial Thermal Processing

Canned (thermally processed) foods are a category of products which receives one of the most extensive heat treatments during processing. Although the temperatures used in thermal processing are not high (110–125°C) compared to many cooking methods (150–300°C), the treatments are often quite long in duration, commonly exceeding 1 hr. Since time, as well as temperature, has been found to influence the extent of mutagen formation during cooking (20,21), it seemed likely that mutagens would be present in these products, especially ones high in protein. If mutagens were found in canned foods, they may pose a risk to consumers because of the quantities of these products being consumed. In the U.S. over 80% of the pink salmon and tuna catches are processed by canning, and nearly 3 billion pounds of canned meats and 1.4 billion pounds of canned fishery products are produced annually (22).

Table 4 shows some canned food products which were found to contain mutagenic substances. Beef and beef-containing products consistently displayed mutagenic activity. However, seafoods were more varied in their mutagenic response. Canned pink salmon was the most mutagenic canned food tested, while tuna (water pack), sardines, and clams contained no, or very low levels of mutagens (16). Basic extracts of canned turkey, chicken, beef stew, ham, Vienna sausages, and corned beef also exhibited mutagenicity less than 2.5 times the spontaneous mutagenicity, as did raw salmon, beef, chicken, and turkey (16).

The reasons for the wide variation in mutagenicity in high protein foods is not clear but is undoubtedly related to differences in their chemical compositions and/or the processing techniques used in each case. For example, it is known that molluscs and crustaceans contain little or no creatine or creatinine (23) and, at least in model systems, these compounds have been found to be important mutagen precursors (23–28). Also, tuna is subjected to a steam precook before being packed into cans and thermal processed. This removes some of the lipids and water-soluble components of the flesh. Salmon, on

the other hand, is placed directly into cans, sealed and processed without any pretreatments.

An important class of processed food (canned products) was thus found to contain mutagens which possess the same extraction behavior (i.e., present in basic organic extracts of food) and *Salmonella* strain specificity as mutagens from grilled or fried meat and fish. Many of the mutagens in cooked foods have been found to be carcinogenic when included in the diets of mice and rats (29–32). While the average mutagenicities detected in canned foods are much lower than those found in many fried or grilled foods, canned foods are widely consumed and, over time, may contribute to any overall risk of consuming the mutagens in heated foods. On the other hand, thermal processing is one of the more important techniques that have been developed for extending the shelf life of perishable foodstuffs, and there is no doubt that thermal processing of foods has increased the quality and variety of foods available to the consumer. It was thus apparent that further studies of mutagen formation in this important category of food products were warranted.

Studies of Mutagen Formation during Thermal Processing

It was presumed that the heat treatment received by the foods during canning was responsible for the appearance of mutagenicity (the raw products contained no mutagens). Thus any parallels that might exist between thermal processing (canning) and the more well studied application of heat during cooking procedures were studied. Frying of meat and fish products usually results in localization of the mutagens near the surface of the product in contact with the heating source (33,34). This possibility was investigated in canned salmon by dividing the contents of the can (1 lb. size) into three portions, a cylindrical core (~5 cm in diameter), the remaining outer cylindrical shell (~1 cm wall thickness) and the broth which was first drained from the can. Each portion was extracted and tested in the Ames assay. It was found that about 76% of the mutagenicity was located in the outer shell (about 50% of total weight can contents) while the core (30% of weight) and broth (20% of weight) contained 17% and 7% of the total mutagenicity, respectively. If an outer shell of smaller wall

Table 5. Effects of reconditioning on mutagenicity of flesh and broth from canned pink salmon.

Sample	Treatment	Revertants/ 80 g equiv. ^a
Canned flesh	Single process	289 ± 34
Canned flesh	Reprocess	611 ± 91
Fluids from canned flesh	Single process	242 ± 27
Fluids from canned flesh	Reprocess	537 ± 46

^a *Salmonella typhimurium* TA 98 plus 80 µL S9 per plate with basic extracts. The means ± SD for four plates are presented.

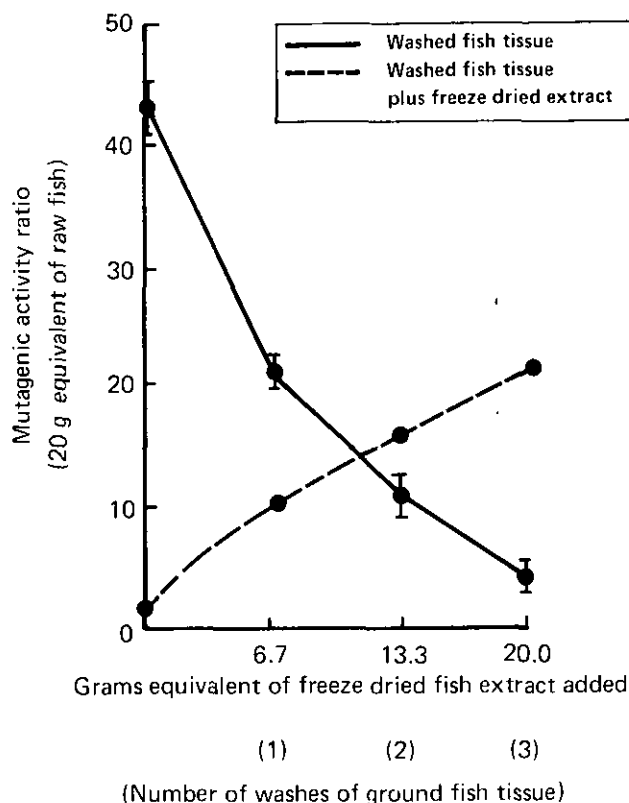


FIGURE 1. Effects of successive washings on mutagenicity of fried ground fish tissue. The restoration of mutagenicity upon addition of increasing amounts of the freeze-dried water extract is also shown.

thickness could be obtained, the differences would probably be even more marked.

Reprocessing

During cooking procedures, an increase in heating time usually results in enhanced mutagen formation. In the canned salmon industry, circumstances arise which require that a product be reprocessed or reconditioned. This obviously results in an increase in total heating time. During reprocessing of salmon the single processed cans are opened, the fluids drained and the flesh inspected and repacked into new cans. Brine or water is then added, the cans sealed, and the heat treatment repeated.

When this procedure was carried out in our laboratory, a twofold increase in mutagen content of the flesh and the drained broth was observed (Table 5). It appeared that the mutagen formation reactions had not gone to completion during the initial heat treatment and, as is the case with cooking, an increase in heating time increased mutagen formation. The fact that mutagenicity of the broth from the single processed fish also increased significantly upon reprocessing suggested

Table 6. Mutagenicity of stock and solids from salmon and ground beef.

Sample	MAR (80 g eq.) ^a	
	Not heat processed	116°C for 85 min.
Ground beef	1.1	3.7
Washed beef solids	0.9	1.4
Beef stock	3.2	9.3
Pink salmon	0.9	4.3
Steamed flesh	1.3	8.7
Salmon stock	1.4	35

^a Mutagenic activity ratio, *S. typhimurium* TA 98 with metabolic activation and the extracts from 80 g of product. The MAR is calculated by dividing the number of revertants on plates containing extracts by the spontaneous reversion rate.

that the mutagen precursors were water-soluble substances that were released from the flesh during the initial heating and reacted to form mutagens during the additional heat processing.

Mutagen Precursors

The water solubility of the mutagen precursors was investigated further in several food systems including canned ground beef and salmon and fried Dover sole. An aqueous extract of Dover sole was prepared by thoroughly blending the ground raw fish flesh with an equal volume of distilled water and filtering through glass wool using slight suction. The retained solids were collected and portions rewashed as above one or two more times. Filtrate from the initial wash of 600 g of fish was freeze-dried. The ground fish flesh and the washed muscle tissue were formed into 60 g, 0.5 to 0.75 cm thick patties and fried (Teflon coated electric skillet, without added cooking oil, at 190°C, 6 min on each patty side). Figure 1 shows that with each successive wash, mutagenicity decreased to about one-half the previous level. Thus the mutagen precursors appear to be substances easily extracted with water from ground fish flesh. To confirm that the precursor compounds were indeed present in the water extract, differing amounts of freeze dried filtrate (dissolved in 15 mL water) were added back to fish flesh which had been washed three times. After thorough mixing, the fish was formed into patties and fried as above. Testing basic extracts of the

Table 7. Effects of freezing and steaming treatments on mutagen formation in canned pink salmon.

Heat treatment	Revertants produced/80 g equiv. ^a	
	Fresh salmon	Frozen, thawed salmon
Steam ^b	10 ± 7	18 ± 32
Can ^c	98 ± 24	270 ± 32
Steam and can	232 ± 37	237 ± 25

^a *S. typhimurium* strain TA 98 with 80 µL S9 per plate and basic extracts.

^b Wrap in foil, steam at 100°C for 1 hr.

^c Place flesh in 307 × 200.25 cans and process in retort for 85 min at 116 °C.

fried product showed an increase in mutagenicity proportional to the amount of freeze-dried filtrate added to the washed tissue (Fig. 1).

The nature of mutagen precursors in two canned food systems was also investigated. Ground beef was treated in a manner similar to the ground sole above and the solids and filtrate (stock) placed in separate cans and heat processed at 116°C for 85 min. For the salmon, steaks of about one-half inch thickness were wrapped in foil, steamed for 1 hr, the fluid which left the tissues during steaming was drained, and the flesh and stock heat processed separately. Table 6 shows that in the case of both the beef and salmon, mutagenicity increased upon heat processing. Especially noteworthy are the high levels of mutagenicity in the beef and salmon stocks.

There were also differences observed between the beef and salmon systems. For example, the washed beef solids exhibited lower mutagenicity than the canned whole beef, as expected, because precursors were removed to the stock. The steamed fish, however, showed an increase over the nonsteamed sample, even though it was evident that, in this case also, some of the precursors were taken from the flesh during steaming (i.e., the drained stock produced mutagens when heat processed). The increased mutagenicity in the steamed flesh may have resulted from conversion of mutagen precursors remaining in the flesh to their more reactive forms. For example, creatine could be converted to creatinine under the steaming conditions (24), thus enhancing the rate of mutagen formation during canning

of steamed versus nonsteamed samples, if creatinine were an important precursor in this system.

It may also be that the steaming disrupted cellular structure within the tissues and facilitated the mixing of mutagen precursors. This possibility is strengthened by the observation that merely freezing and then thawing the flesh prior to canning also led to a similar difference between the fresh and pretreated (frozen) canned product. Freezing and thawing also disrupts membranes and intracellular organelles, and denatures proteins. Combined freezing and steaming prior to canning did not further increase the mutagen content (Table 7).

Model System Studies

The preceding experiments and the test of reprocessing suggested that a fish broth or stock might serve as a model system to further investigate mutagen formation during thermal processing or frying. Table 8 illustrates the results of studies which used fish extract model systems and various additives, and pH adjustments to help characterize the types of reactions involved in mutagen formation. The fish extract used for the fried Dover sole model system was prepared by blending ground fish tissue with equal volumes of distilled water, filtering and rewashing the solids once. The combined filtrates were heated to near 100°C for 20 min, and the heat precipitated proteins removed by centrifugation at 5000 rpm for 10 min. The supernatant was filtered through Whatman No. 1 filter paper and used in the model system studies. Preliminary heating studies with this Dover sole supernatant and the residue from centrifugation (heat precipitated proteins) showed that only the supernatant contained compounds capable of producing mutagens when heated. This method for production of the supernatant is very similar to that used by Taylor et al. (35,36) to obtain a soluble beef supernatant (S₂) for a model beef boiling system.

To simulate the conditions that exist during frying, the sole supernatant was first reduced in volume by heating in an open beaker at temperatures less than 100°C until the moisture content was 45 to 53%. This is near the moisture content ($49 \pm 3.8\%$) found in the crust of ground beef fried on six different surfaces (20). This residue was then heated for 12 min in a 150-mL beaker placed in an oil bath maintained at 130°C.

A more simple scheme was used to produce the canned salmon model system. Pink salmon flesh was heated to about 90°C for 1 hr in a volume of distilled water equal to the weight of the flesh. The mixture was filtered through glass wool and Whatman No. 1 filter paper, the filtrate placed in cans, in some cases including the additives listed in Table 8, sealed, and heat-processed at 116°C for 85 min.

Adjustment of the pH of the salmon stock prior to canning had varied effects. Decreasing the pH to 4.6 (from an initial pH 6.6) did not alter the overall mutagenicity, while buffering at pH 8.0 appeared to cause a

Table 8. Mutagen formation in canned salmon and fried sole model systems.

Additive	MAR ^a	
	Canned salmon system	Fried sole system
None ^b	2.0*	2.5*
Heat only	15.6	45.3
Buffer at pH 4.6	17.8	NT ^c
Buffer at pH 8.0	9.6*	NT
Ascorbic acid (1%)	1.8*	NT
Nitrite (125 ppm)	4.7*	NT
Ribose		
(1% salmon, 0.1% sole)	11.4	74.7*
Dihydroxyacetone (0.1%)	NT	80.5*
Creatine		
(0.7% salmon, 0.1% sole)	27.1*	47.9
Alanine (0.1%)	NT	44.3
Sodium bisulfite (0.5%)	0.4*	2.5*
Sodium borohydride (0.5%)	NT	3.9*

^a Mutagenic activity ratios for basic extracts from 80 g salmon stock or the equivalent of 100 g raw Dover sole. *S. typhimurium* strain TA 1538 with 80 μ L S9 per plate.

^b Unheated salmon stock or evaporated sole supernate prior to 130°C treatment. All other samples were heated as follows: salmon stock placed in cans with additives, sealed and heated in retort for 85 min at 116°C; evaporated sole supernate (45–53% moisture), heated at 130°C for 12 min.

^c NT = not tested.

* Significantly different than heated only sample ($p = 0.01$).

decrease. It would seem from this data that mutagen formation reactions were favored at pH's less than neutrality. If Maillard-type browning were the only reactions involved in mutagen formation, the above observations would probably be unexpected. Taylor and co-workers (35) have reported that there are two pH optima for mutagen formation in a boiled beef model system, one at pH 4 in the acidic region, and one at pH 9 at alkaline pH. This may also be the case for the canned salmon model system and is one area that requires further investigation.

Addition of 1% ascorbic acid decreased mutagen formation significantly in the canned salmon model system. Ascorbic acid (AA) is a carbonyl compound which can itself participate in browning type reactions. In fact, the samples with AA added were visibly more brown in color than the control samples. AA is also a mild reducing agent and an acid. Its influence on mutagen formation does not seem to be through its acidic properties since the pH of the salmon stock with 1% AA was 4.4, near that where the buffering with phosphate did not change mutagenicity. Recently Namiki and Hayashi

(37) reported on a novel pathway to browning in the Maillard reaction, one involving free-radical formation in the very early stages of browning, prior to Amadori rearrangement. They showed that AA could, through its reducing activity, enhance the formation of the important free radical intermediate, an *N,N'*-disubstituted pyrazine. Nitrite is also a reducing agent and its influence on mutagen formation may be through this same mechanism.

Some browning reaction intermediates do enhance mutagen formation in model systems. It has been suggested that 2-methylpyridines may be incorporated into the quinoline portion of IQ and MeIQ (2-amino-3,4-dimethylimidazo[4,5-f]quinoline) and 2,5-dimethylpyrazine may form part of the quinoxaline of MeIQx (24). If the free-radical pathway which is encouraged by AA were followed and mutagens similar to those mentioned above formed, they would contain alkyl substituents on the nitrogens of the quinoxaline portion of the molecule. However, mutagens of this configuration have not yet been identified. Thus, the *N,N'*-disubstituted pyrazines may not be as readily converted to mutagens as some other browning reaction intermediates and AA and nitrite may be decreasing mutagenicity by diverting mutagen precursors through a pathway, which creates non-mutagen forming intermediates.

The addition of ribose and dihydroxyacetone to the fried sole system produced a significant increase in mutagenicity, while ribose supplementation of the canned salmon system had little effect (Table 8). This disparity is difficult to explain unless the limiting mutagen forming reactants are different in these two products. This conclusion is supported by the fact that creatine resulted in enhanced mutagen formation in the salmon system but not in the sole supernate.

One major difference between salmon and sole is in their lipid contents; the sole used in this study contained 0.7% lipid while the salmon, a moderately fatty fish, was about 5% lipid. The lipids in marine fishes contain numerous highly unsaturated fatty acids that are prone to undergo autooxidation. This oxidation process can produce numerous carbonyl products including various aldehydes, ketones and enols (38).

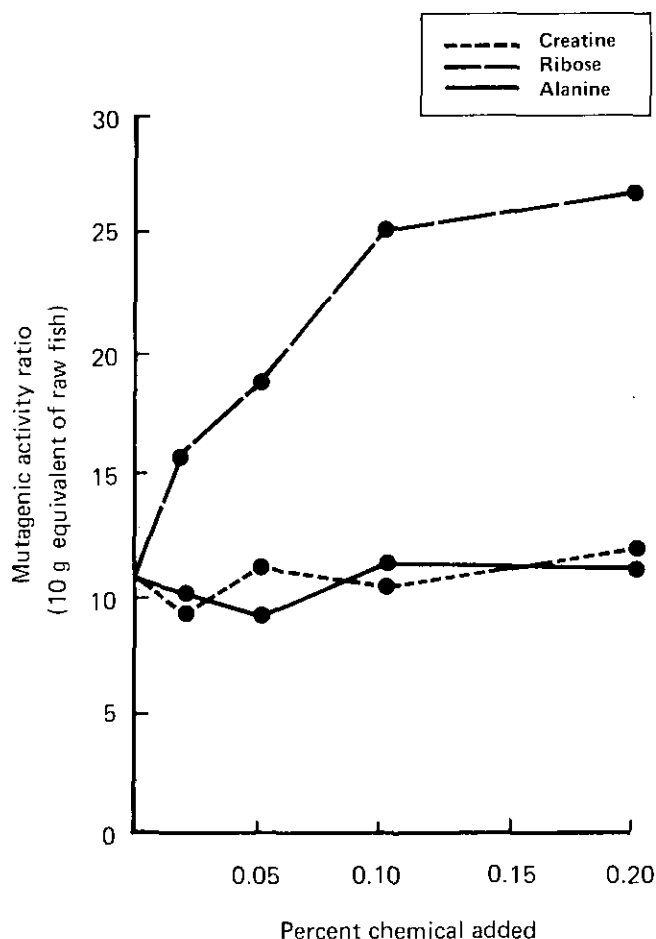


FIGURE 2. Dose-response relationships for addition of ribose, alanine and creatine to the fried Dover sole model system.

Table 9. Effect of Maillard browning inhibitors on mutagen formation in whole foods.

Treatment prior to heating	MAR ^a	
	Canned pink salmon ^b	Fried sole fillets ^c
None	8.9	49.6
0.5% added NaHSO ₃	1.2	8.0 ^d
5% NaHSO ₃ dip	3.9	11.5
20 min soak 5% NaHSO ₃	—	6.6
1% added ascorbic acid	3.8	—

^a Mutagenic activity ratio for basic extracts from 80 g canned salmon or 20 g fried sole using *S. typhimurium* TA 98.

^b Canning at 116°C for 85 min.

^c Fry at 6 min per side at 190°C.

^d Ground sole patties plus bisulfite.

Pokorny (39) showed that browning reactions could proceed in model systems containing proteins and unsaturated lipids from fish, both with and without water being present. Under conditions similar to roasting and frying, the lipids were found to decompose and be accompanied by the formation of brown pigments. Deep fat frying of fish in thermally oxidized cooking oils led to a greater loss in available lysine in the fish than frying in fresh oils (40). The decrease in available lysine was thought to be due to browning type reactions. Fatty acids can produce mutagenic substances when heated with creatine and amino acids (41), and these mutagens were found to form at temperatures as low as 100°C with the more highly unsaturated lipids producing the highest levels. Thus lipids or their breakdown products may be as, or more, important than sugars in the mutagen formation reactions in salmon.

It was found that the enhancement of mutagen formation by ribose in the sole model system was limited to the concentration range 0.02 to 0.1% of added ribose with amounts greater than 0.1% producing no further increase in mutagen formation (Fig. 2). It thus appears that other necessary reactants in the system were exhausted and became limiting at the higher ribose concentrations. Figure 2 also shows the lack of effect of added alanine or creatine in the fried sole system.

Taylor et al. (28) found that while additional creatine phosphate and tryptophan enhanced mutagen formation in their beef model system, other amino acids and sugars had little effect. Iwaoka et al. (42,43) also showed that ammonium ions added to aqueous extracts of a baked high carbohydrate product (biscuits) led to mutagen production, while this was not the case for a high protein product (ground beef).

Overall, these data suggest that differences in the relative concentrations of carbonyl and nitrogenous compounds present in various foods play a role in the generation of different types and levels of mutagens during heating. They also suggest that Maillard type reactions may be involved in mutagen formation and browning reaction inhibitors might diminish the levels of mutagens. As seen in Table 8, the introduction of NaBH_4 or NaHSO_3 , both of which interact with carbonyl groups to block browning reactions (44,45), eliminated mutagen formation in the model systems, thus supporting the above conjecture.

The model systems also showed that all the components necessary for mutagen formation were water-soluble, heat-stable, polar substances that could easily be washed from fish tissues. These studies pointed out that mutagens can form in foods heated at low temperatures, around 115°C, which is lower than those used in most studies of cooked foods and even most model systems. Mutagens were also shown to form at the high moisture levels found within canned food systems. This is in contrast to most cooked foods where it has been shown that mutagen formation usually accompanies the dehydration which occurs at the surface of foods during frying, grilling or roasting.

The effectiveness of browning reaction inhibitors in whole food systems was also examined. As little as 0.2% sodium bisulfite thoroughly mixed into ground sole decreased the mutagenicity of fried patties by 50% and at a level of 1% eliminated mutagen formation. Sodium bisulfite also reduced the mutagenicity of canned salmon and fried sole fillets when applied by various other means (Table 9). Thus the model systems can provide a simple method for evaluating techniques (such as bisulfite addition) which may modify mutagen formation during heating of foods.

However, model systems do not always duplicate exactly the processes occurring in whole foods. In very simple model systems (26,27), combining creatine, glucose and glycine, MeIQx(2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline) was found to be the major mutagen formed (90%), with small quantities of 3,7,8-diMeIQx also present (10%). When threonine was used in place of glycine, twice as much 3,4,8-diMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline) was formed as MeIQx (46). In food-grade beef extract, which is produced by concentrating the water-soluble components of beef tissue, MeIQx comprised about 65% of the mutagenicity with the remainder being contributed by IQ (13). In contrast, a model beef boiling system produced IQ, Trp-P-1 (3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole) and Trp-P-2 (3-amino-1-methyl-5H-pyrido[4,3-b]indole) in varying proportions depending

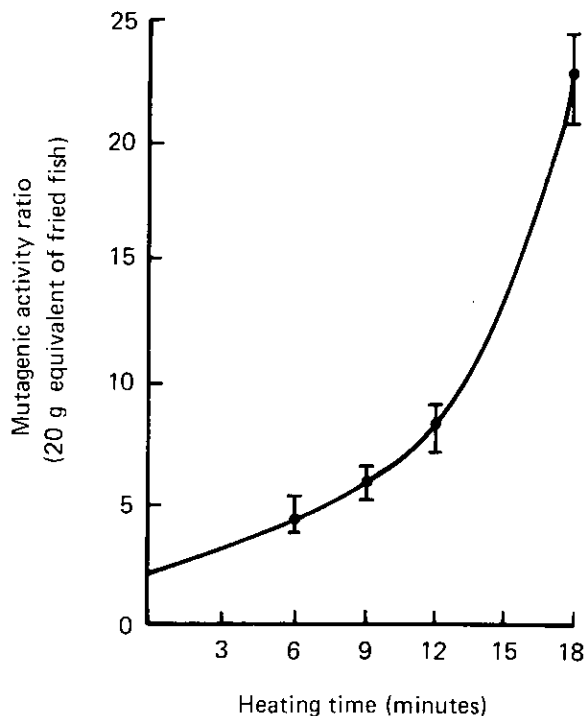


FIGURE 3. Dependence of mutagen formation on time in a fried Dover sole model system at 125°C. Mutagenic activity ratios are for basic extracts of the heated model system using *S. typhimurium* strain TA 1538 with metabolic activation.

on supplementation of the system with creatine phosphate, tryptophan and FeSO_4 (28). MeIQx did not appear to form under the conditions used (100°C).

In fried beef, a major mutagen produced is MeIQx (25% to 30% of total mutagenicity), with significant amounts of diMeIQx and smaller quantities of IQ (4–12%) present depending on temperature of heating (13,47). Felton et al. (47) also found that at least 10 other mutagens were present in fried beef. Thus as tempting as it may be to equate mutagen formation in model systems to that in whole foods, the complex nature of the physical and chemical processes taking place during heating makes this difficult at present.

Effects of Time and Temperature on Mutagen Formation

Time and temperature both play roles in the mutagen formation in model systems and during cooking of foods. At frying temperatures from 150°C to 300°C, mutagenicity initially increased rapidly in ground beef and then reached a plateau (20,21). A similar pattern was shown to occur during the frying of sole fillets (48). Bjeldanes (20) showed by multiple regression analysis that tem-

perature was a much more important variable than time in describing mutagen formation in fried hamburgers.

Taylor (35) investigated the time dependence of mutagen formation in a boiled beef system and found an exponential increase in mutagenicity over a 30-hr boiling time. This same nonlinear curve was observed for heating the fried sole supernate system at 125°C for times up to 18 min (Fig. 3). When a semilogarithmic plot of the data in Figure 3 was made, a straight line ($r = 0.984$) was obtained (not shown).

A quantitative measure of the temperature dependence of mutagen formation in this fried sole system was also examined. Heating for 12 min at temperatures from 100 to 150°C produced a linear relationship when the log revertants produced per time per gram of product was plotted versus the reciprocal of absolute temperature (an Arrhenius plot). Strictly speaking, in order to apply the Arrhenius equation, the temperature of the reaction mixture should be used, rather than the temperature of the heating medium. The fried sole system simulates the phenomena occurring at the surface of fried products. The moisture content of the evaporated sole supernatant which was heated at the various temperatures listed above averaged 49%, similar to that found in the crusts of fried ground beef. The evaporated supernate residue was spread thinly in beakers during heating, and it was found that during heating in this manner, the residue increased in temperature rapidly and soon reached a plateau, the relative level of which corresponded to the temperature of the heating medium. This probably explains why a linear relationship ($r = 0.975$) was observed in the Arrhenius plot (Fig. 4), even though the temperature of the heating medium was used rather than the actual temperature of the system (which was available for only a few of the eight temperatures used in the heating experiment).

Canned solid products like salmon are heated primarily by conduction and the thermal conductivity of these types of products is rather low (49). The surface heat-transfer coefficient for processing in condensing steam is, however, large in comparison. Thus the temperature of the product near the can wall rapidly approaches the temperature of the heating medium and remains there throughout the process time. This is a major factor in the observation that excessively high processing temperatures will cause severe degradation of the food near the container wall long before the food at the center of the can has reached the process temperature (50). Vitamin and amino acid destruction has been shown to occur to a greater extent in the outer compared to the inner portions of the canned product (51,52), and data presented in an earlier section also showed that mutagen levels were higher in the parts of the product nearest the can wall. These phenomena probably contribute to the linear Arrhenius plot ($r = 0.996$) obtained when mutagenicity data for canned salmon were plotted versus process temperature and not product temperature (Fig. 4). The activation energies derived from the slopes of the regression lines

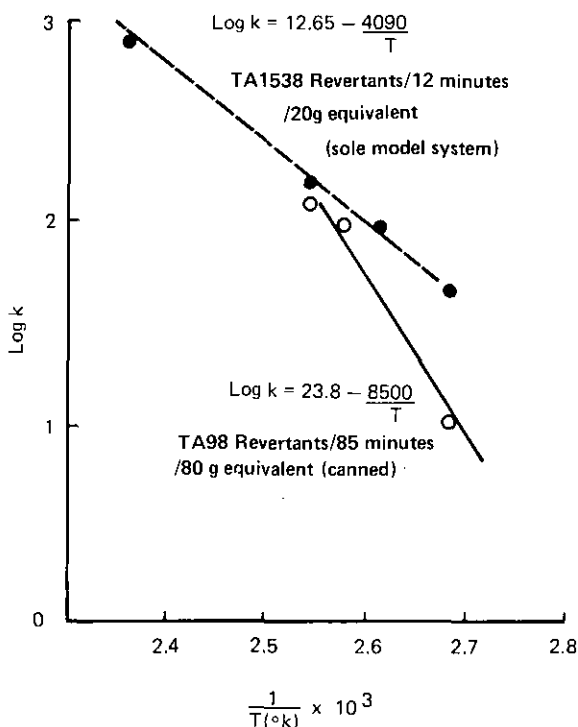


FIGURE 4. Arrhenius plots of mutagen formation in a fried Dover sole model system and in canned pink salmon as a function of temperature. The Arrhenius equation in the form, $\log k = \log A - (E_a/2.303RT)$, allows the activation energy (E_a) to be estimated from the slope of the least-squares regression line through the plotted points. In this equation, k is the rate of mutagen formation in revertants produced/time/gram-equivalent, T is absolute temperature, R is the gas constant (1.99 cal/mole), and A is a constant characteristic of the reaction.

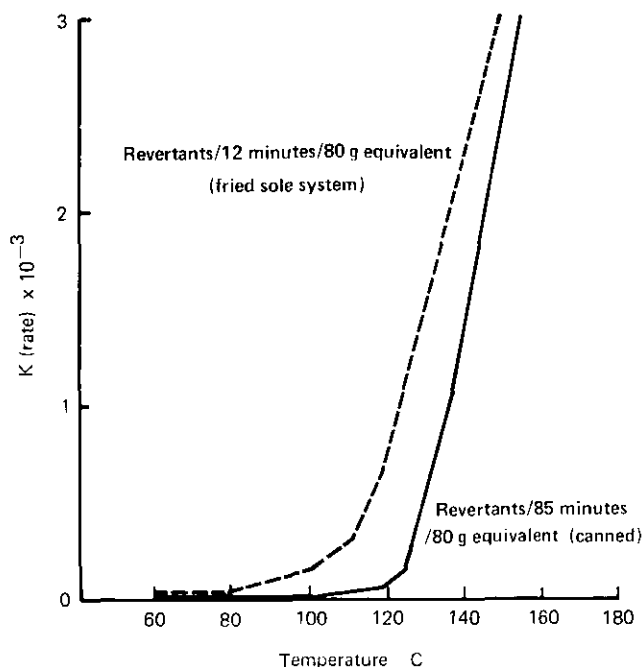


FIGURE 5. Theoretical rates of mutagen formation at different temperatures for canned pink salmon and a fried Dover sole model system.

through the points were found to be 39 kcal/mole for canned salmon and 19 kcal/mole for the Dover sole system.

Using these E_a values, theoretical rates of mutagen formation at higher temperatures were calculated and are shown graphically in Figure 5. These data seem to indicate that heating at temperatures above about 140°C would lead to dramatic increases in mutagen formation. This does seem to be the case for fried ground beef (20,21). For the thermal processing of salmon (and most conduction heating high protein products), the highest retort temperatures used are generally not greater than 125°C (53) for the reasons mentioned above (nutrient destruction and scorching of the product near the can wall).

Figure 5 also suggests that mutagenicity should not be significantly different for the range of processing temperatures (110°C to 125°C) established by National Food Processors Association (53) for these types of

products. Canned pink salmon was thus prepared using three recommended processes (53) of equivalent lethality: 110°C for 139 min, 116°C for 85 min, and 121°C for 64 min. As seen in Table 10, there was no difference in mutagen formation during these widely different processes even though the time varied by over a factor of two, while the temperature was changed by only 10°C. As mentioned earlier, temperature was found to be a more important variable than time in mutagen formation during cooking (20). This finding also seems to be borne out in the canned food system.

The above experiments on thermal processing involved metal cans as containers. An increase in the use of retort pouches can be expected in the future because of their advantages in lower weight and energy savings. It is interesting to note that the only retort pouch product which was examined in our studies (beef stew) contained significantly higher levels of mutagenicity than a similar product in a standard metal can. The higher surface-to-volume ratio for products packaged in retort pouches may contribute to the difference. Recently the FDA (54) has increased the maximum retort temperatures at which laminates can be used from 121°C to 135°C. The possibility that mutagen formation might be significantly increased by this change, as suggested by the above kinetic studies, should certainly be investigated.

Characterization of Mutagens in Canned and Fried Pink Salmon

Before any decisions on the risk or safety of consuming a mutagen containing product can be made, the identity of the compounds responsible for the mutagenicity must be known. In the case of most mutagens formed during heating of foods the levels are quite low, usually low or less than parts per billion, and this makes the above task very difficult.

To obtain information on the mutagens present in heated pink salmon, basic extracts of both the canned and fried product were fractionated using HPLC and C_{18} , cyano, and amino columns. In both products, three major fractions from the initial C_{18} column separation possessed mutagenic activity. One fraction exhibited a

Table 10. Effects of process time and temperature on mutagen content in canned pink salmon.

Temperature, °C	Time, min	MAR ^a
100	139	12.4 ± 0.8
116	85	10.2 ± 1.6
121	64	10.1 ± 1.8

^aMAR = number of revertants on plates containing fish extract divided by the spontaneous rate. *S. typhimurium* TA 98 with 80 μ L S9 per plate was used with the basic extracts from 80 g of product.

Table 11. Relative proportions of the three major mutagens in canned and fried pink salmon.

Product	% of total mutagenicity ^a		
	Mutagen 1 ^b	Mutagen 2 ^c	Mutagen 3 ^d
Canned salmon	39	35	25
Fried salmon	24	67	9

^a*S. typhimurium* TA 98 with S9.

^bFractions eluting between 18 and 22 min. during separation of basic extracts on C_{18} column (0.46 cm ID × 25 cm, 10 μ m particle diameter) and a methanol/water gradient; 30% methanol/70% water increased linearly to 80% methanol in 50 min.

^cFractions eluting between 26 and 30 min.

^dFractions eluting between 52 and 58 min.

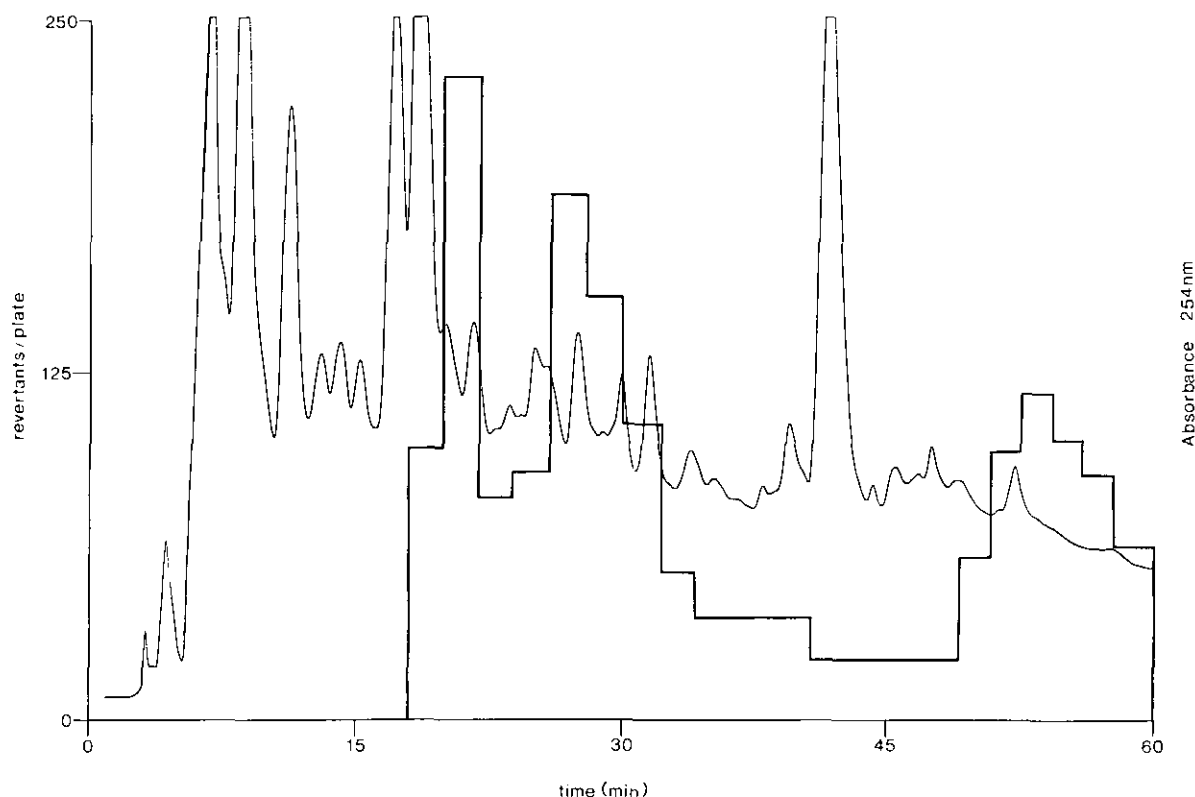


FIGURE 6. Chromatogram of C_{18} HPLC separation of a basic extract from canned pink salmon with bar graphs showing the mutagenicity of fractions (0.5% of each fraction per Ames test plate, spontaneous revertants have been subtracted). The retention times of IQ, MeIQ and MeIQx were 20 ± 0.2 , 23 ± 0.6 , and 24.5 ± 0.9 min, respectively.

retention time in the same range as IQ while the other two fractions were somewhat more nonpolar than the IQ type mutagens (Fig. 6). The contribution to total mutagenicity was approximately equal among the three fractions of the canned product; however, the mutagen with intermediate retention time comprised about two-thirds of the total mutagenicity in the fried product. It appeared to form at the expense of both other mutagens (Table 11).

Each of the three fractions separated by the initial high pressure liquid chromatography was further purified by additional HPLC on C_{18} and cyano and/or amino columns. After each stage of HPLC purification, portions of each fraction were treated with nitrite or hypochlorite (55,56) and again tested for mutagenicity. The mutagens in all fractions showed the typical patterns of the IQ type of mutagens, that is, resistance to nitrite inactivation and total loss of mutagenicity upon exposure to hypochlorite (Table 12).

Even though 14 kg of canned salmon had been used as starting material for mutagen isolations, the amount of material remaining after secondary HPLC fractionation was too small for further spectral characterization of the compounds. Therefore, fried salmon, in which mutagen formation is about 30 times greater, was also

used as a source of mutagenic compounds. Basic extracts of commercially canned pink salmon produced about five *S. typhimurium* TA 98 revertants per gram of original product, while four separate lots of fried pink salmon produced 177 ± 39 TA 98 revertants per gram of fried fish. Since the mutagen with intermediate retention time comprised 65% of mutagenicity in fried salmon, the most information was obtained on this substance. After sequential separations on C_{18} (twice) and cyano columns, a UV absorption peak which corresponded to the mutagenicity peak was observed during a final amino column separation. The fractions containing mutagenicity were combined and the UV spectrum in methanol exhibited a weak absorption maximum at 278 nm. It was evident from the direct probe mass spectrum (MS) of these combined fractions, however, that several different compounds were present.

The MS fragmentation pattern exhibited significant substituted aromatic hydrocarbon character (benzenes and naphthalenes) but also contained a prominent peak at $m/e = 224$. The intensities of the $m/e = 223$ and $m/e = 225$ peaks were approximately 78% and 23%, respectively, of the intensity of the $m/e = 224$ peak. Felton and co-workers (57) have reported the presence of a mutagen of mass 224 with UV maximum at 277 nm

Table 12. Effects of nitrite and hypochlorite treatments on the mutagenicity of IQ, MeIQ, MeIQx, Glu-P-2 and Trp-P-2, and mutagens from canned and fried pink salmon.

Sample	% of control (mean and range) ^a	
	Nitrite (20 mM)	Hypochlorite (0.018%)
Glu-P-2	2.9 (2.4–3.4)	1.1
Trp-P-2	3.3 (1–5)	0.9
2-Aminoanthracene	2.5 (1–4)	1.3
IQ	87 (76–98)	3.1
MeIQ	55 (44–74)	1.5
MeIQ _x	91 (87–95)	4.0
Basic extract (fried)	89	12
Mutagen 1 fried ^b	75	9.0
Mutagen 2 fried ^b	80	12
Mutagen 3 fried ^b	84	25
Mutagen 1 fried ^c	87	9.0
Mutagen 2 fried ^c	83	9.0
Mutagen 1 fried ^d	—	—
Mutagen 2 fried ^d	84 (73–95)	5.0
Mutagen 3 fried ^d	78	3.4
Basic extract (canned)	78	66
Mutagen 1 (canned) ^d	63	21
Mutagen 2 (canned) ^d	84	21
Mutagen 3 (canned) ^d	90	20

^a TA 98 revertants on plates with treated sample (nitrite or hypochlorite treatment) divided by TA 98 revertants on plates with same amount of untreated sample times 100. Each determination consisted of the mean of two plates for each treatment.

^b Fractions which contained mutagenicity after the initial C₁₈ HPLC fractionation.

^c Fractions containing mutagenicity after secondary C₁₈ HPLC fractionation.

^d Fractions containing mutagenicity after final amino column HPLC purification.

in fried beef. It may be that the major mutagen in fried salmon is the same as one of the mutagens detected in fried beef.

In any event, none of the three prominent mutagens in canned or fried salmon exhibited HPLC retention time patterns which matched those of the reference compounds IQ, MeIQ, or MeIQ_x, and the nitrite sensitivity was different than the pyrolysis type mutagens. Thus it appears that the major mutagens in heated salmon are different than the prominent ones in fried ground beef, where MeIQ_x was found to be the most abundant mutagen (47). Since the concentrations of free amino acids, sugars, and creatine do not appear to be markedly different in beef, pork, poultry, or fish muscles, some other minor component seems to be responsible for the differing rates and types of mutagens formed in these different food products.

Summary

We can conclude that, of the major industrial food processing techniques in common use, commercial ther-

mal processing or canning appears to be most likely to produce mutagens. We have seen that browning type and possibly other classes of reactions contribute to mutagen formation during thermal processing, and that mutagens can form at the relatively low process temperatures and high moisture levels found in canned products. The model systems proved to be a valuable technique not only for investigating the chemical processes involved in mutagen formation, but also for evaluation of strategies for reducing mutagenicity in whole food products.

And finally, it should be noted that the levels of mutagenicity found in even the most mutagenic canned products are 2 to 15 times lower than the majority of fried or grilled high protein foods. In the case of cooked products, the consumer has the option of using lower temperature cooking methods such as steaming or microwave to reduce or eliminate mutagen formation. However, this option is not available for consumers of canned foods, unless they choose not to purchase the product. This represents a significant difference between cooked and commercially processed foods, and brings us to some final questions for consideration. These relate to the issue of regulating the mutagens formed during thermal processing, especially if the mutagens are found to be carcinogens. If these substances are to be regulated, what form should the regulations take? Should these compounds be considered naturally occurring or added carcinogens? Should the canning process be regarded in the same manner as food irradiation? Currently the U.S. Food and Drug Administration has no definite rules governing this situation, and it will be interesting to observe how the food industry and regulatory agencies address these concerns in the future.

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